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FGF signals from the nasal pit are necessary for normal facial morphogenesis

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ABSTRACT

Fibroblast growth factors (FGFs) are required for brain, pharyngeal arch, suture and neural crest cell development and mutations in the FGF receptors have been linked to human craniofacial malformations. To study the functions of FGF during facial morphogenesis we locally perturb FGF signalling in the avian facial prominences with FGFR antagonists, foil barriers and FGF2 protein. We tested 4 positions with antagonist-soaked beads but only one of these induced a facial defect. Embryos treated in the lateral frontonasal mass, adjacent to the nasal slit developed cleft beaks. The main mechanisms were a block in proliferation and an increase in apoptosis in those areas that were most dependent on FGF signaling. We inserted foil barriers with the goal of blocking diffusion of FGF ligands out of the lateral edge of the frontonasal mass. The barriers induced an upregulation of the FGF target gene, *SPRY2* compared to the control side. Moreover, these changes in expression were associated with deletions of the lateral edge of the premaxillary bone. To determine whether we could replicate the effects of the foil by increasing FGF levels, beads soaked in FGF2 were placed into the lateral edge of the frontonasal mass. There was a significant increase in proliferation and an expansion of the frontonasal mass but the skeletal defects were minor and not the same as those produced by the foil. Instead it is more likely that the foil repressed FGF signaling perhaps mediated by the increase in *SPRY2* expression. In summary, we have found that the nasal slit is a source of FGF signals and the function of FGF is to stimulate proliferation in the cranial frontonasal mass. The FGF independent regions correlate with those previously determined to be dependent on BMP signaling. We propose a new model whereby, FGF-dependent microenvironments exist in the cranial frontonasal mass and caudal maxillary prominence and these flank BMP-dependent regions. Coordination of the proliferation in these regions leads ultimately to normal facial morphogenesis.

Introduction

During ontogeny of the face, several initially separate buds of mesenchyme covered in epithelium known as the facial prominences grow out and merge together to give rise to the upper and lower jaws. The majority of the mesenchyme within the facial prominences is derived from cranial neural crest cells, with a smaller contribution from paraxial head mesoderm. The neural crest derived mesenchyme gives rise to all of the bone and cartilage in the face (Couly et al., 1996; Couly et al., 1993; Kontges and Lumsden, 1996; Noden, 1983). The majority of skeletal patterning information is carried in the neural crest-derived mesenchyme (Schneider and Helms, 2003; Tucker and Lumsden, 2004). Soon after neural crest cell migration ends, the facial prominences form around the primitive oral cavity. The frontonasal mass lies in the midline and is flanked by the nasal pits, the lateral nasal prominences are between the nasal pit and the eye, the maxillary prominence are at either side of the oral cavity, whereas the mandibular prominences lie below the maxillary prominences. The upper jaw is formed by the frontonasal mass with contributions from the maxillary and lateral nasal prominences. In contrast, the lower jaw is formed entirely by the mandibular prominences. To form the upper beak there must be contact between the corners (globular processes) of the frontonasal mass and maxillary prominences. Following growth and contact of the two prominences, a bilayered epithelial seam is formed and then is removed through apoptosis and epithelial–mesenchymal transformation (Sun et al., 2000). The degrading epithelium is invaded by mesenchyme from either side and residual grooves are filling out by proliferation.

Even though the bigger aspects of jaw identity are established prior to the formation of facial prominences (for example, distinguishing upper versus lower jaws), refinement of the basic pattern is still required to give species-specific morphology. Work carried out in avian embryos has shown that expression of certain growth factors such as Bone Morphogenetic Proteins (BMPs) is correlated with differences in beak shape (Abzhanov et al., 2004; Wu et al., 2004). For example, the shape of the early frontonasal mass is thought to be closely related to the final shape of the upper beak. The mechanism for modifying frontonasal mass morphology is thought to be by the positive influences of growth factors on proliferating cells within the

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facial mesenchyme (Wu et al., 2006, 2004). This idea is supported by the demonstration in several studies that antagonism of BMP signaling with Noggin reduces the proliferation and consequently the size of facial prominences (Ashique et al., 2002a; Foppiano et al., 2007; Wu et al., 2006, 2004).

In addition to BMPs, several other growth factor families are known to be important for growth of facial prominences. Wingless-related proteins (Wnt) constitute one class of signal required for frontonasal mass growth. In experiments where embryos were exposed to *Dkk1*, an antagonist of Wnt signaling, frontonasal mass growth was inhibited and clefts occurred (Brugmann et al., 2007). The epithelially-expressed gene *Sonic Hedgehog* is required at two times in development, early on to establish the facial midline and later to promote outgrowth of the frontonasal mass (Hu and Helms, 1999; Hu et al., 2003). BMPs are required to establish the *SHH* expression domain in the caudal edge of the frontonasal mass (Foppiano et al., 2007).

The contribution of FGF signaling to midfacial growth and the fusion of the upper lip has been addressed in several conditional deletions of *Fgf8*. In several lines, the mutant embryos have a truncated face (Macatee et al., 2003) and mandible (Trumpp et al., 1999), suggesting that FGF signaling may be required at specific times in development. In addition, there are intriguing data suggesting that prolonged expression of *FGF8* and maintenance of proliferation at higher levels at the edges of the frontonasal mass is one of the reasons why the duck has a wider beak than the chicken (Wu et al., 2006). However no one has rigorously studied the role of FGFs in facial morphogenesis.

There are 22 FGF ligands in mammals (Zhang et al., 2006), five of which are expressed in the mouse face *Fgf2*, *Fgf8*, *Fgf9, Fgf10, Fgf17* and *Fgf18* (Bachler and Neubuser, 2001; Crossley and Martin, 1995; Havens et al., 2006; Karabagli et al., 2002; Kettunen and Thesleff, 1998; Rice et al., 2004). In the chicken genome only 5 of these genes are present, including *FGF2, FGF8, FGF9, FGF10* and *FGF18*. Of these genes, *FGF2* is ubiquitously expressed, and the others are mainly expressed in the superficial ectoderm surrounding the nasal slit and lining the maxillo-mandibular cleft (Havens et al., 2006; Karabagli et al., 2002; McGonnell et al., 1998; Ohuchi et al., 2000; Richman et al., 1997). FGFs bind to three FGF receptors in the facial mesenchyme. *FGFR1* is expressed ubiquitously whereas *FGFR2*, is expressed in the medial frontonasal mass mesenchyme while *FGFR3* is restricted to the caudal edge of the frontonasal mass and medial edges of the maxillary prominences (Matovinovic and Richman, 1997; Wilke et al., 1997).

Our study focuses on the control of facial morphogenesis using gain or loss of function approaches that target the FGF pathway. Since there are so many FGF family members, to decrease signaling, we implanted beads soaked in a pan-antagonist of FGFRs, SU5402 (Mohammadi et al., 1997). To increase FGF receptor activation FGF2 protein was applied to discrete regions of the face. Our results revealed several novel FGF-dependent and independent regions in the frontonasal mass and maxillary prominence that together coordinate growth and contact of the facial prominences. Finally, to determine where the FGF signal originated, we implanted foil barriers to block directional signaling in the frontonasal mass.

Methods

Bead implantations and foil implants

White leghorn eggs were obtained from the University of Alberta and incubated at 38 °C. SU5402 (SUGEN, USA and EMD Biosciences, UK; Mohammadi et al., 1997) was dissolved in DMSO (dimethyl sulfoxide). AG1X2 beads (Formate form, Biorad) were soaked in 5 μl of SU5402 for 1 h with a drop of 0.01% Fast Green added for bead visualization. FGF2 beads were prepared by soaking 200–300 μm Affigel beads with either 1 mg/ml FGF2 or FGF8b protein (Peprotech). Control beads were soaked in DMSO or buffer. Beads were inserted into small incisions into the facial prominences and the final position was recorded.

Aluminium foil was inserted medial to the nasal slit in the frontonasal mass. Care was taken to position the foil cranially and not to interfere with the globular process.

Skeletal preparation and skull analysis

To study bone and cartilage morphology Hamburger and Hamilton stage 37–39 (E12–14) embryos were stained as described (Plant et al., 2000). Each process of the maxilla, premaxilla and palatine bones was compared to the normal, contralateral side. Bony processes were scored as normal, reduced or absent. The reduced category included bony processes that were greater than 50% shorter in length. The effect of SU5402 bead position on skeletal morphology was determined using χ^2 analysis (significance level set at $p<0.05$).

Fluorescence photography of whole heads and analysis of early phenotypes with FGF2 beads

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight, stained in 1:5000 dilution of SybrSafe (Invitrogen) in PBS for 10 min, rinsed and photographed using the GFP filter on a Leica MZFLIII microscope.

BrdU analysis

Approximately 50 μl of 10^2 M BrdU (bromodeoxyuridine) was injected into the heart 2 h prior to collection. Embryos were fixed 12 h after bead implantation. Wax sections (7 μm) were treated with proteinase K, exonucleases and then incubated with neat primary antibody (Amersham, GE Healthcare). Alexa Fluor 488 (Molecular Probes, Invitrogen) labeled goat anti-mouse antibody (1:50) was incubated at room temperature for 30 min. Slides were coverslipped using Prolong Gold Antifade with DAPI (Molecular Probes, Invitrogen).

Proliferation index was calculated by dividing the number of BrdU positive cells by the DAPI positive (total cell number). Sections at least 14 μm apart were chosen so that different populations of cells would be represented. We used two WCIF Image J plugins for automated cell counting: ITCN (Image-based Tool for Counting Nuclei) for the BrdU positive cells and the Nucleus Counter for the DAPI stained nuclei.

To determine if there were differences in cell proliferation related to bead position we divided the lateral frontonasal mass into thirds. The cranial region lined up with the top edge of the nasal slit, the caudal region included the corner or globular process of the frontonasal mass. Each area was approximately 200 μm wide by 100 μm tall and contained approximately 800 cells. We compared each region counted, bead position and compared SU5402 treatment to DMSO using multifactorial ANOVA (MANOVA) with Fisher Least Significant Difference post hoc testing ($p<0.05$; Statistica). In the FGF2-treated embryos, changes in proliferation were qualitatively compared to the contralateral side or Tris-treated controls.

Cell death analysis

For Nile Blue Sulfate staining, bead implantation was performed without neutral red staining and embryos were collected 3, 6, and 9 h (Song et al., 2004). For TUNEL, embryos were collected 6 and 16 h after bead implantation and sections were stained as described (Buchtová et al., 2007). Apoptotic bodies in nearly adjacent sections were counted in the same regions that were used in the BrdU analysis plus an additional region in the medial frontonasal mass. Since apoptosis results in loss of cell integrity we placed specimens into one of three categories: 0–5; 6–10; 11–50 apoptotic bodies (Table S3).

Whole mount in situ hybridization and immunohistochemistry

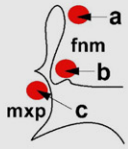
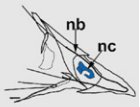
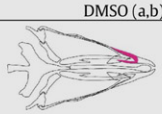
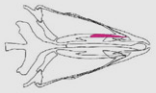
Whole mount in situ hybridization was performed as described (Song et al., 2004). Section in situ hybridization was performed with antisense ^{35}S-labeled antisense probes as described (Wilke et al., 1997). The following individuals generously provided gallus cDNAs: G. Martin, *SPRY2*; P. Francis-West, *BMP4*; M. Kessel, *DLX5*; S. Wedden, *MSX1, MSX2*; O. Pourquie, intronic and exonic *FGF8*; S. Keyse, *PYST1*. The *SPRY4* probe was obtained from the MRC Geneservice (UK; Clone ID: 603786019F1).

Embryos for wholemount phosphorylated MAPK staining were stained as previously published (Corson et al., 2003) with the Phospho-p44/42 Map Kinase antibody (Cell Signaling #9101; 1:350 dilution). An additional proteinase K step for antigen retrieval was included.

Results

Our study uncovered differential gene expression patterns within the frontonasal mass and other regions of the face that led us to hypothesize there were differences in FGF signaling within the developing face. In order to understand the endogenous FGF signals taking place at the time of fusion we used bead implantation to locally block or stimulate FGF signaling. We focused our study on stage 26–28 chicken embryos, a time when key differences in proliferation have been noted (MacDonald et al., 2004; McGonnell et al., 1998; Wu et al., 2006, 2004). These stages are also just prior to the major morphogenetic changes such as fusion of the lip and beak outgrowth. Our

Table 1
Effects of blocking FGF signaling vary according to bead position

Stage of surgery	Initial location of bead	External notch	Nasal bone	Nasal conchae
				
		% with an external cleft	% with reduction	% misshapen
26	a	40% (18/45)	0 % (0/45)	56% (25/45)
26	b	0% (0/16)	0 % (0/16)	0 % (0/16)
26	c	0% (0/15)	nd	0% (0/15)
26	DMSO (a,b)	0 % (0/6)	0 % (0/6)	0 % (0/6)
			Premaxillary bone	
		% with reduction in the size of the nasal process and body of premaxilla	% missing either of the maxillary or palatal process	
26	a	93% (40/45)	62% (28/45)[a]	
26	b	86% (12/14)	0 % (0/16)	
26	c	0 % (0/15)	0 % (0/15)	
26	DMSO (a,b)	0 % (0/6)	0 % (0/6)	
			Palatine process of maxillary bone	
		% with reduction	% dislocated	
26	a	60% (27/45)[b]	44% (20/45)	
26	b	0 % (0/16)	0 % (0/16)	
26	c	0 % (0/15)	0 % (0/15)	
26	DMSO (a,b)	0 % (0/6)	0 % (0/6)	
			Palatine bone	
		% with reduction		
26	a	58% (26/45)[c]		
26	b	0 % (0/16)		
26	c	0 % (0/15)		
26	DMSO (a,b)	0 % (0/6)		

[a] 6 are moderately reduced.
[b] 31 have ppmx dislocated proximally.
[c] 10 are severely truncated.

findings with blocking FGF receptor signaling show a surprising degree of dependence on FGF signals in some areas of the face and not in others. We also demonstrate through the use of barriers that it is likely an FGF signal coming from the nasal pit that is required for growth and skeletal patterning of the facial midline.

Loss of FGF signaling in the FNM induces a cleft phenotype — a notched upper beak

We placed beads soaked in SU5402 into three positions in the frontonasal mass and one in the maxillary prominence. Specifically we targeted regions on either side of the fusion zone to determine whether FGF signaling was required for lip fusion. Embryos were treated at stage 26, approximately 16 h before epithelial contact in the fusion zone takes place. There is also rapid proliferation within the facial prominences at these stages, especially in the lateral edges of the frontonasal mass (MacDonald et al., 2004; McGonnell et al., 1998; Wu et al., 2006, 2004). We first tested a variety of soaking concentrations to determine the minimum concentration that would lead to reproducible defects (Table S1). Based on these dose–response experiments we selected 10 mg/ml as the optimal soaking concentration. Some embryos treated in the lateral edge of the frontonasal mass with 10 mg/ml SU5402 developed cleft beak morphology (n=13/45) whereas those with beads placed in the midline of the frontonasal mass (n=14/14, data not shown) or the anterior maxillary prominence developed normally (n=15/15; Table 1). The placement of DMSO-soaked beads did not affect morphogenesis (n=6/6).

In the next series of experiments we focused more on the lateral edge of the frontonasal mass since this an area adjacent the FGF-rich nasal slit epithelium. Beads were placed at opposite ends of the frontonasal mass, cranial and in line with the superior edge of the nasal slit or at the caudal edge, in the globular process. We found that significant numbers of embryos treated with cranial beads, developed an external cleft (n=18/45; Table 1; Fig. 1A arrowhead). Skeletal analysis revealed that several specimens with a normal external appearance actually had defects in the bones supporting the edge of the beak. In those embryos there was a gap between the premaxillary and maxillary bones (20/45; Table 1; Figs. 1B–D) caused by a reduction in size of the premaxillary bone (the maxillary process, nasal process were reduced in 28/45 and 40/45 embryos respectively). Often, the nasal conchae were unsupported and extended outside the nasal capsule (n=25/45; Figs. 1B–D). The overall length of the upper beak and growth of the midline prenasal cartilage was unaffected.

We were surprised to find that all embryos treated in the globular process had normal upper beaks (globular process, n=16/16, Figs. 1E–G; Table 1) even though there is expression of *FGF8* in this region.

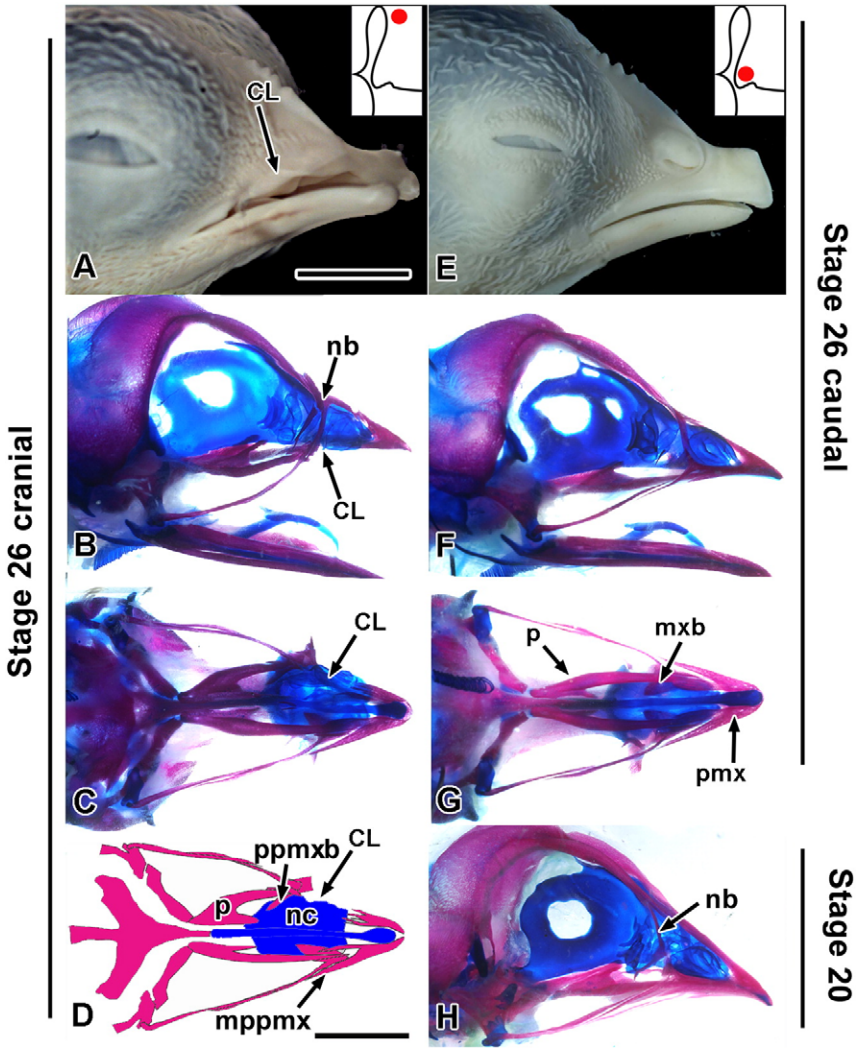


Fig. 1. Effects of SU5402 on beak morphology. External views (A, E) or stained skeletons (B, C, F–H) of stage 38 embryos originally treated at stage 26 or 20 with a bead soaked in 10 mg/ml of SU5402. (A–D) Embryos tread with cranial beads (inset in panel A) have notch in the tomium or cleft beak (CL). (B, C) Sagittal and palatal views of the beak. There is a cleft between the maxillary and premaxillary bones, deviations of the upper beak towards the treated side however beak length is normal. In addition the maxillary bone is shifted proximally and the nasal conchae extend beyond the confines of the nasal cavity. Panel D is a camera lucida drawing of the specimen in C illustrating the bony morphology. (E–G) The caudal bead position did not affect development. Beak morphology is normal externally (E) and internally (F, G). (H) Sagittal view of an embryo treated with bead placed in the frontonasal mass at stage 20 (inset in panel E). The nasal bone is absent and nasal conchae are reduced on the treated side. Frontonasal mass derivatives are normal. The lower beak has been removed. Scale bar = 5 mm. Bar in D applies to B, F, C, G, H. Key: CL — cleft, mppmx — maxillary process of the premaxilla, mxb — maxillary bone, nb — nasal bone, nc, nasal conchae, p — palatine bone, pmx — premaxillary bone, ppmxb — palatine process maxillary bone.

These functional blocking experiments revealed for the first time that FGF signaling was dispensable in the two areas of the face that must meet for lip fusion to occur: the globular process and medial maxillary prominence.

The lack of effect on outgrowth of the upper beak by beads placed into the centre or side of the frontonasal mass could indicate that there are other times in development when there is a greater dependence on FGF signaling for outgrowth of the upper beak. At stage 20 there is strong expression of *FGF8* across the superficial ectoderm of the entire frontonasal mass, and then it is downregulated approximately 6 h later leaving only the transcripts surrounding the nasal pit (Song et al., 2004). In order to determine whether stage 20 embryos were dependent on FGF signaling, we placed beads soaked in SU5402 into the frontonasal mass. Instead of clefts or the predicted beak truncations, the treatment with SU5402 led to slight deviations of the prenasal cartilage and deletions of the nasal bone (Fig. 1H, $n = 11/22$). The effects on the nasal bone are in agreement with previous studies where we have shown that FGFs are required for lateral nasal prominence development at stage 20 (Song et al., 2004). The maxillary and palatine processes of the premaxillary bone were also reduced in size ($n = 8/22$). The lack of clefts at stage 20 versus stage 26 indicates the changing requirement for FGF signalling as the face develops. We went on to focus on stage 26 embryo treated with SU5402, since phenotypes were connected to an important aspect of facial morphogenesis, fusion of the prominences. The data on differential responsiveness to SU5402 in cranial and caudal bead positions lead us to hypothesize that there were also differential gene expression changes in FGF targets.

SU5402 blocks FGF signaling and decreases expression of FGF targets within the frontonasal mass

In order to confirm whether SU5402 had decreased endogenous FGF signaling more in the cranial frontonasal mass than the caudal, we examined expression of three genes known to be directly regulated by FGF ligands, *SPRY2, SPRY4* and *PYST1* (Chambers et al., 2000; Eblaghie et al., 2003). *SPRY2* is induced within 25 min of FGF application (Chambers et al., 2000). Another family member, *SPRY1* can be

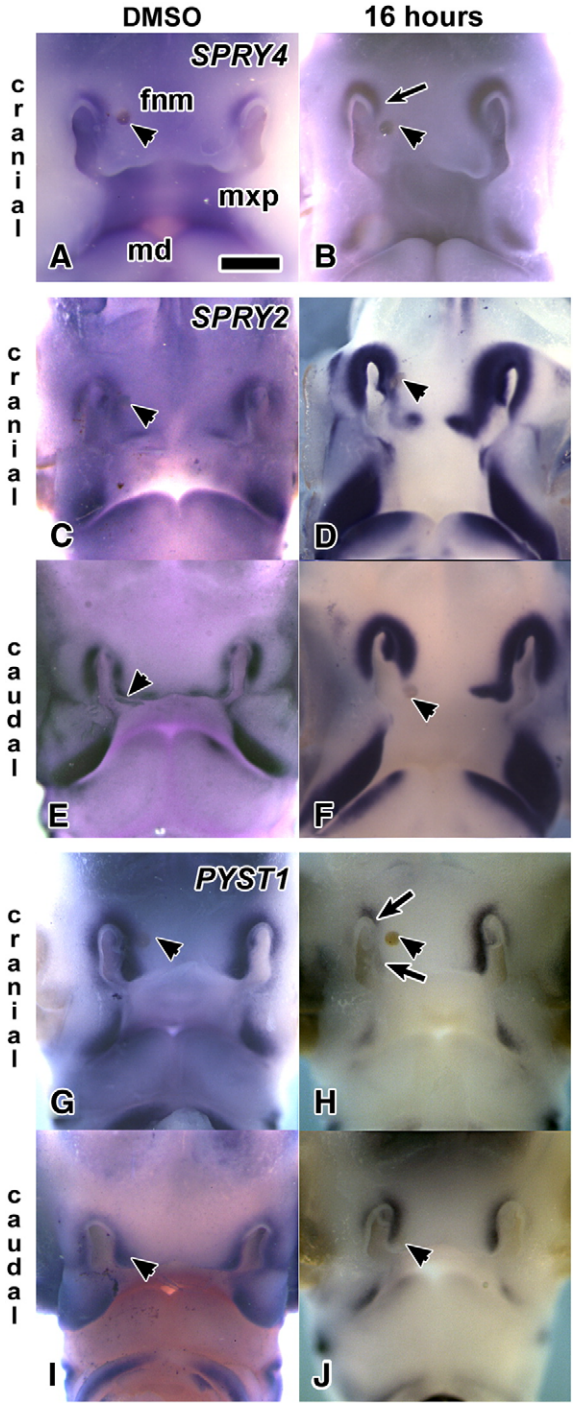


Fig. 2. FGF signaling antagonists are differentially affected by cranial and caudal SU5402 bead positions. Wholemount in situ hybridization of embryos fixed 16 h after beads soaked in 10 mg/ml of SU5402 or DMSO were implanted. Position of beads is shown with black arrowheads in all panels. (A, C, E, G, I) DMSO bead does not affect expression. (B) *SPRY4* and (D, F) *SPRY2* expression are decreased in the lateral edge mesenchyme of the frontonasal mass. Caudal beads (F) have a more local effect on expression than cranial beads (D) where signal is reduced from the bead down to the globular process. (H) Ablation of *PYST1* expression with a cranial bead. (J) *PYST1* expression is locally decreased surrounding the caudal bead. Scale bar = 500 μm. Key: fnm, frontonasal mass; md, mandibular prominence; mxp, maxillary prominence.

induced in the presence of cycloheximide by FGF8 (Liu et al., 2003). These studies suggest that the SPRY family members are direct targets of FGF signaling. In addition to be induced by FGFs, SPRY proteins act in a negative feedback loop to inhibit FGFR signaling by preventing the downstream phosphorylation cascade (Kim and Bar-Sagi, 2004). During normal embryo development it is likely that SPRY proteins maintain FGF homeostasis in areas where there are high levels of FGF ligands. There is a close correlation between areas that express high levels of *FGF8* and those that express *SPRY2* (Chambers and Mason, 2000). We also found the same relationship between *SPRY2* and *FGF8* in the face (Figs. 2C, E and data not shown). *SPRY4* was expressed in the almost the same areas as *SPRY2* but frontonasal mass transcripts for *SPRY4* were mainly concentrated at the cranial end of the nasal slit (Fig. 2A).

We examined expression at 6 h to increase the probability of identifying direct targets and at 16 h, to determine the duration of gene expression changes. Beyond 16 h, many of the cranially treated specimens had deficiencies in the frontonasal mass, the first indication that a growth disturbance had occurred. Both *SPRY4* and *SPRY2* expression were reduced in embryos with cranial beads 6 h after beads were implanted (data not shown; Table S2). Sixteen hours after cranial bead implantation, expression of *SPRY4* had partially recovered near the cranial edge of the nasal slit (Fig. 2B; Table S2). Less recovery of *SPRY2* was seen within the globular process itself where expression continued to be weaker compared to the non-treated side ($n=7/7$; Fig. 2D). The more robust recovery in some parts of the face could be due to higher endogenous FGF levels in the regions near the nasal slit. Indeed we found that SU5402 treatment did not decrease *FGF8* transcripts (Table S2) so once the antagonist had cleared from the tissue, we assume that the translated protein could resume normal function.

In contrast, caudal beads decreased *SPRY2* but the effects were confined mainly to the globular process at both 6 and 16 h after implantation (6 h, $n=7/7$; 16 h, $n=6/6$; Fig. 2F, Table S2). The caudal beads lay outside the expression domain of *SPRY4*, and therefore there was no effect on expression (6 h, $n=4/4$; 16 h, $n=10/10$; data not shown). We did not examine *SPRY1* in our studies, because it has nearly an identical expression pattern to *SPRY2* (data not shown) and similar responsiveness to FGFs (Liu et al., 2003).

As an alternative to SPRY genes we also looked at expression of another antagonist of FGF signaling: PYST1. PYST1 dephosphorylates the activated threonine and tyrosine residues on ERK2 (Camps et al., 1998), thereby also dampening the FGF signalling cascade (Eblaghie et al., 2003). *PYST1* is expressed in similar areas to *SPRY2* in the frontonasal mass and maxillary prominence (Figs. 2G–J). Similar to *SPRY2, PYST1* expression was largely ablated in the frontonasal mass at both time points in cranially treated embryos (Fig. 2H; Table S2). The caudal beads caused a local decrease in *PYST1* expression mainly restricted to the globular process at both time points (Fig. 2J; Table S2). Overall, there were surprising long-distance effects on expression of *SPRY2* and *PYST1* from cranial beads whereas beads in the globular process did not exert their effects in a cranial direction.

The decrease in transcripts for three antagonists of FGF signaling, *SPRY2*, *SPRY4* and *PYST1* could cause a disregulation of the downstream signalling cascade and a net increase in FGF signalling. To determine whether or not this was true we looked at the amount of phosphorylated ERK in the frontonasal mass with immunohistochemistry. Embryos with cranial beads had decreased staining along the lateral edge of the frontonasal mass ($n=4/4$, Fig. S1B), compared with a localized decrease surrounding the caudal bead position ($n=4/4$, Fig. S1C). The effects on *SPRY2*, *PYST1* and phospho-ERK support the conclusion that there is a net decrease in the level of FGF signaling caused by SU5402 implants, which results in cleft lip.

To determine whether transcription factors required for facial morphogenesis were affected by SU5402 we look at expression of *MSX1* and *MSX2*. Both genes are downstream of FGF (Kettunen and Thesleff, 1998; Mina et al., 2002) and BMP signaling (Ashique et al., 2002a; Mina et al., 2002). Furthermore *MSX2* is more highly expressed in the globular process while *MSX1* is found in the entire lateral edge of the frontonasal mass. In cranially-treated embryos there was decreased expression of *MSX1* along the lateral edge of the frontonasal mass including the globular process (6 h, $n=4/4$; Figs. 3A, B; Table S2)

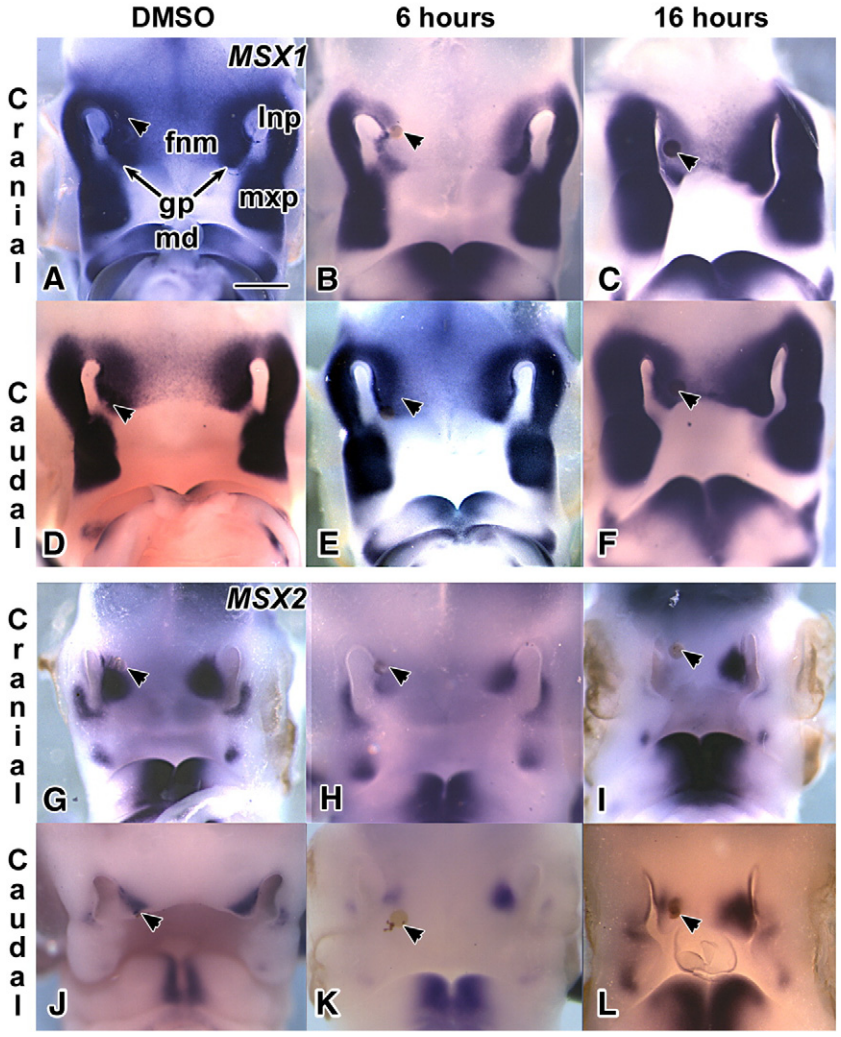


Fig. 3. *MSX1* and *MSX2* expression are reduced in response to SU5402. Wholemount in situ hybridization in embryos treated with DMSO or SU5402-soaked beads (10 mg/ml). (A, D, G, J) Expression domains are unaffected in controls. *MSX1* and *MSX2* expression is rapidly downregulated in cranial implants (B, H) and remains low 16 h later (C, I). (E, F, K, L) Caudal bead treatment greatly reduced expression near the bead. Scale bar=500 μm. Key: fnm, frontonasal mass; gp, globular process; lnp, lateral nasal process; mdp, mandibular prominence; mxp, maxillary prominence.

however by 16 h expression had recovered (n=4/4; Fig. 3C; Table S2). Caudal bead implants had little effect on expression at both timepoints (16 h: n=5/5; Figs. 3D–F).

MSX2 was greatly reduced following cranial SU5402 treatment (n=5/5 for both time points, Figs. 3H, I). Globular process beads had an even greater effect, either completely eliminating or significantly reducing expression (6 h, n=6/7, Fig. 3K; 16 h, n=6/6, Fig. 3L). This is consistent with the fact that beads were placed directly into the area of highest gene expression. The decrease in expression of FGF targets further illustrate that FGF signalling is reduced and not essential for lip fusion in the globular process.

FGF signaling in the cranial frontonasal mass is required to maintain cell proliferation and cell survival

We hypothesized that the skeletal phenotypes were produced by changes in cell proliferation and apoptosis and that these cellular dynamics would vary in the two different bead positions. Several studies have reported that the lateral edge of the frontonasal mass has higher proliferation compared to the centre (MacDonald et al., 2004; McGonnell et al., 1998; Wu et al., 2006, 2004). However, no study had previously compared proliferation in the globular process to cranial regions of the lateral frontonasal mass.

On qualitative analysis of DMSO control or normal embryos, we noted a striking absence of proliferating cells in the globular process mesenchyme (Figs. 4A–F and data not shown, n=21/21). Other observations were that immediately adjacent to the globular process there were paired areas of high proliferation and in between these regions in the centre of the frontonasal mass there was low proliferation. In addition, the maxillary mesenchyme directly opposite from the globular process had lower proliferation (n=17/17). These patterns of proliferation were consistent in treated and untreated sides of the face (Figs. 4C–F, H) as well as in DMSO controls (Figs. 4A, B). Quantitative analysis of proliferation along the edge of the frontonasal mass supported these observations (caudal third had significantly lower proliferation than the cranial third, $p<0.05$; Fig. 4G). This suggests that instead of acting as the leading edge, the globular process is pushed or displaced towards the maxillary prominence by the proliferation of more cranial mesenchyme.

In treated embryos, those with cranial beads had significantly decreased proliferation in the cranial two-thirds of the frontonasal mass compared to the same region in DMSO controls ($p<0.05$; Figs. 4E, G). The overall cell density was similar in treated and control embryos. The caudally-implanted SU5402 did not alter proliferation in the caudal region even though the beads were directly in contact with these cells (Figs. 4B, H). One explanation is that these cells are not

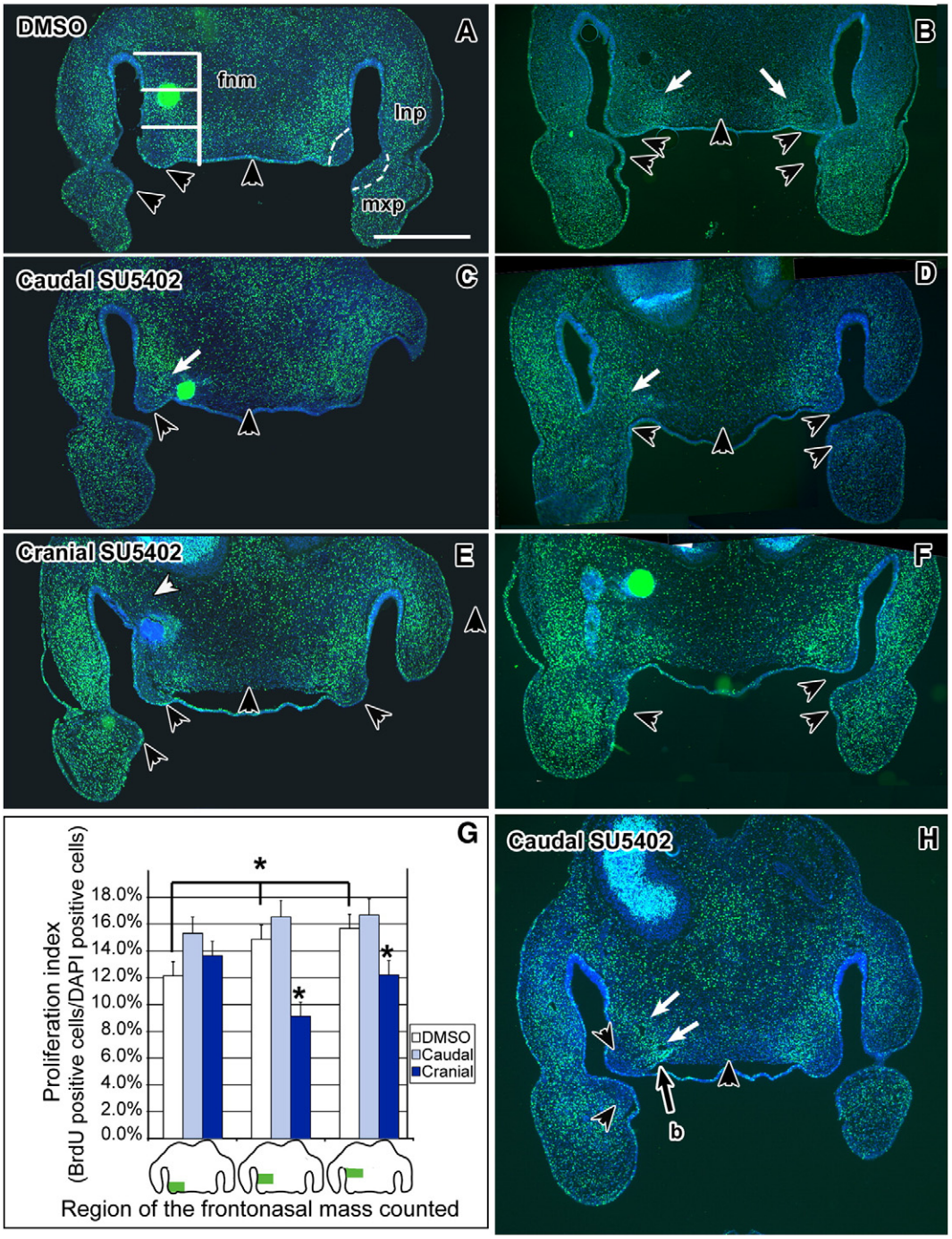


Fig. 4. Cell proliferation is selectively reduced in cranially treated embryos. Immunofluorescence detection of BrdU antibody in frontal sections. Specimens were pulse-labeled for 2 h with BrdU and fixed 12 h after bead implantation with 10 mg/ml SU5402 or DMSO. Proliferating cells are green and nuclei are blue. Black arrowheads show low proliferation and white arrows show high proliferation in all sections. Two sections from each specimen, shallow in the left panel and deeper in the right panel are represented in panels A–F. (A, B) Control specimen showing normal patterns of proliferation. Lower proliferation is seen in the globular process and cranial maxillary prominence (dashed lines in panel A) as well as in the centre caudal edge of the frontonasal mass. A band of proliferating cells is seen immediately medial to the low proliferation zone of the globular process (white arrows in B). Areas counted are indicated with white grid in A. (C, D) No qualitative decrease in proliferation is seen. Areas of high proliferation (white arrows) are still present even though the bead is located very close by. (E, F) Decreased proliferation caused by SU5402 (white arrowhead in (panel E). (G) Relative rate of proliferation in the regions indicated in panel A. Asterisk over white bars show caudal third of the frontonasal mass has significantly lower proliferation than the middle or cranial third. Asterisks on dark blue bars show significantly lower proliferation in cranially-treated embryos compared to the other treatments for a given region. (H) A different caudally-implanted specimen than in panel C, panel D showing caudal proliferation is not reduced although the bead is right next to this region (white arrows). Bead is not in this section but position in adjacent section is noted with arrow. Scale bar = 500 μm; Key: b, bead; fnm, frontonasal mass; lnp, lateral nasal prominence; mxp, maxillary prominence.

dependent on FGF signalling for their proliferation. The absence of changes in the BrdU data could explain why globular process was unaffected by SU5402 beads. In addition these data support the novel idea that there is an FGF-dependent growth centre located near the cranial-end of the nasal slit.

FGF is known as a cell survival factor and targeted deletion of *FGF8* in the face results in increased apoptosis (Trumpp et al., 1999). There is normally no apoptosis along the lateral edge of the frontonasal mass (McGonnell et al., 1998), except for low levels within the globular process (Ashique et al., 2002a). With Nile blue staining, we observed

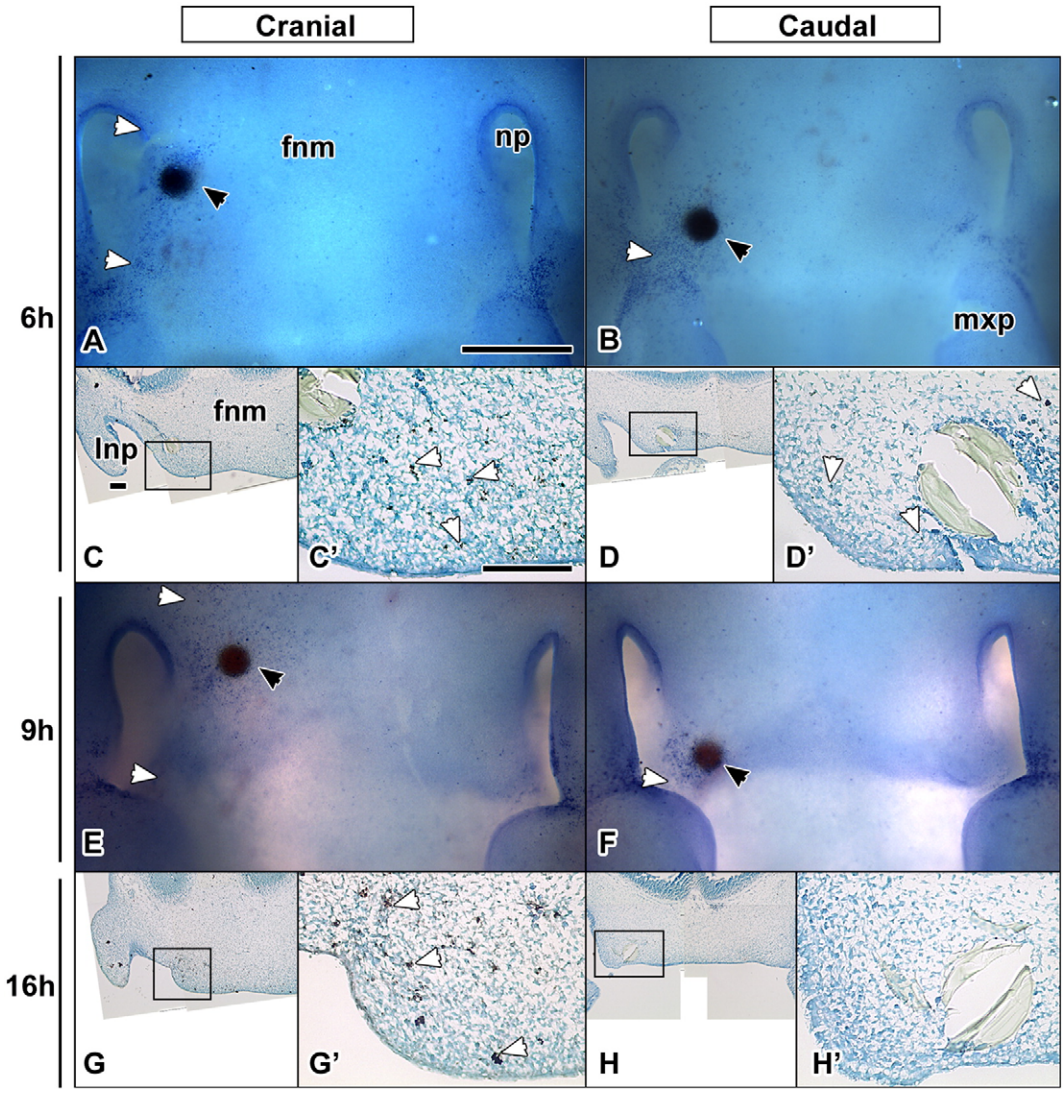


Fig. 5. Cell death is increased with SU5402. Embryos treated with 10 mg/ml SU5402 soaked beads. (A, B, E, F) Nile Blue staining. (C,D,G,H) TUNEL staining. White arrowheads indicate apoptotic cells. (A) The cranial bead increased cell death along the edge of the nasal pit into the globular process. (B) Increased cell death near the caudal bead. Normal areas of cell death are present in the groove between the lateral nasal and maxillary prominence. (C, C′) Mesenchymal cell death is induced by the cranial bead but only background levels are seen in caudal bead treatment (arrowheads in panel D′). (E) More extensive cell death extending to the telencephalon (upper arrowhead) is induced by the cranial bead. (F) Very localized apoptosis surrounding the caudal bead (arrowheads). (G, G′) Persistent mesenchymal apoptosis is observed after cranial bead treatment. (H, H′) No detectable signal in the lateral mesenchyme of the frontonasal mass. Scale bars: A = 0.5 mm, applies to panels B, E, F; C = 100 μm and applies to panels D, G, H; C′ = 100 μm and applies to panels D′, G′, H′. Key: fnm, frontonasal mass; lnp, lateral nasal prominence; mxp, maxillary prominence; np, nasal pit.

increased cell death on the treated side of the FNM, confirming that the effects of SU5402 are unilateral. Similar to the long distance effects on gene expression, cranial beads increased cell death from the cranial margin of the nasal slit down to the globular process (3 h: $n=5/7$, not shown; 6 h, $n=6/6$, Fig. 5A; 9 h, $n=3/4$ Fig. 5E). Elevated apoptosis in the mesenchyme was confirmed in sections (Table S3; Figs. 5C, C′). In contrast, the caudal bead position slightly enlarged the normal area of cell death found in the globular process of the frontonasal mass (3 h: $n=6/7$, not shown; 6 h: $n=5/5$, Fig. 5B; 9 h $n=4/4$, Fig. 5F). We found that mesenchymal apoptosis had returned to normal levels 16 h after caudal treatment (Figs. 5H, H′). There was no change in apoptosis in any of the DMSO treated embryos demonstrating that the surgery itself did not stimulate cell death (data not shown).

The nasal slit provides necessary FGF signals for frontonasal mass morphogenesis

Through inserting SU5402 beads into the mesenchyme we have shown that FGF signals from the mesenchyme are required to promote cell survival and cell proliferation. However, the close proximity of the beads to the nasal slit may have meant that we also interfered with signals from this tissue. To determine whether FGF signalling within the nasal slit was affected, we looked at expression of *DLX5* a transcription factor localized to this epithelium (Figs. S2A, D). Cranial SU5402 decreased expression of *DLX5* ($n=6/6$; Figs. S2A–C; Table S2) providing the first evidence that SU5402 is affecting the adjacent epithelium.

In order to see whether the nasal slit epithelium could be providing FGF to the mesenchyme we did two types of experiments. The first was an extirpation experiment where as much of the olfactory epithelium as could be removed was stripped from the nasal slit. We then looked for changes in expression of *PYST1*. In sections of embryos we found that the olfactory epithelium was partially removed with this procedure ($n=18/18$, 0 and 6 h; Figs. S2E, F) and lead to a unilateral decrease in *PYST1* ($n=5/8$, Figs. S2G, H). These data suggest that in addition to blocking mesenchymal FGF signalling with SU5402 we may have also reduced FGF signalling originating in the nasal slit epithelium.

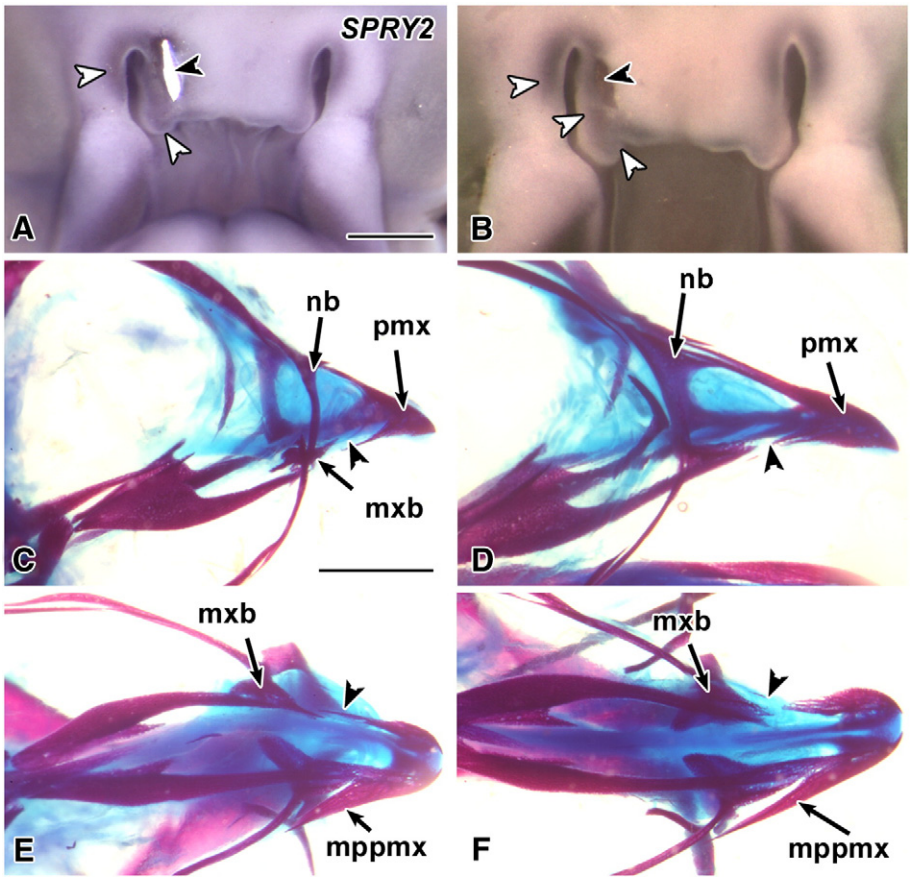


Fig. 6. Effects of foil barriers inserted into the frontonasal mass. (A, B) embryos fixed 16 h after foil barrier implantation demonstrate that the position of the foil is just medial to the *SPRY2* expression domain, leaving a small margin of mesenchyme between the foil and the nasal slit. In both embryos there is increased expression on the treated side of the face, on the frontonasal mass and lateral nasal side of the nasal slit. The embryo in B had the foil removed before photography. (C, E) sagittal and ventral views showing the defect in the premaxillary bone. This embryo also has a reduce nasal bone on the treated side (C). (D, F) Sagittal and ventral views of a different embryo with similar defects. The nasal bone is of normal size in this embryo. Scale bars = 500 μm for panels A, B and 2 mm for panels C–F. Key: mppmx, maxillary process of premaxilla; nb, nasal bone; mxb, maxillary bone; pmx, premaxilla.

We next placed foil barriers in the lateral edge of the frontonasal mass, medial to the *SPRY2* expression domain at stage 25. We have already shown that *SPRY2* is sensitive to levels of FGF signaling and therefore selected this gene to obtain a readout of whether FGF levels had changed in the frontonasal mass as a result of putting in the foil. We also targeted the foil barrier to the cranial half of the nasal slit, an area determined with our bead experiments to be dependent on FGFs and medial to the *SPRY2* expression domain. We wanted to determine whether FGFs would accumulate in the facial mesenchyme adjacent to the nasal slit with a barrier in place. We found that *SPRY2* was increased in the lateral edge of the frontonasal mass in a majority of embryos (n=9/11, Figs. 6A, B). This corroborates with the previously described directional effects of cranial SU5402 beads. An unexpected result was the upregulation in the lateral nasal prominence (n=7/11, Figs. 6A, B). The data suggests that the foil barrier resulted in the amplification of FGF signaling and it was high enough to trigger the upregulation of *SPRY2* in this region.

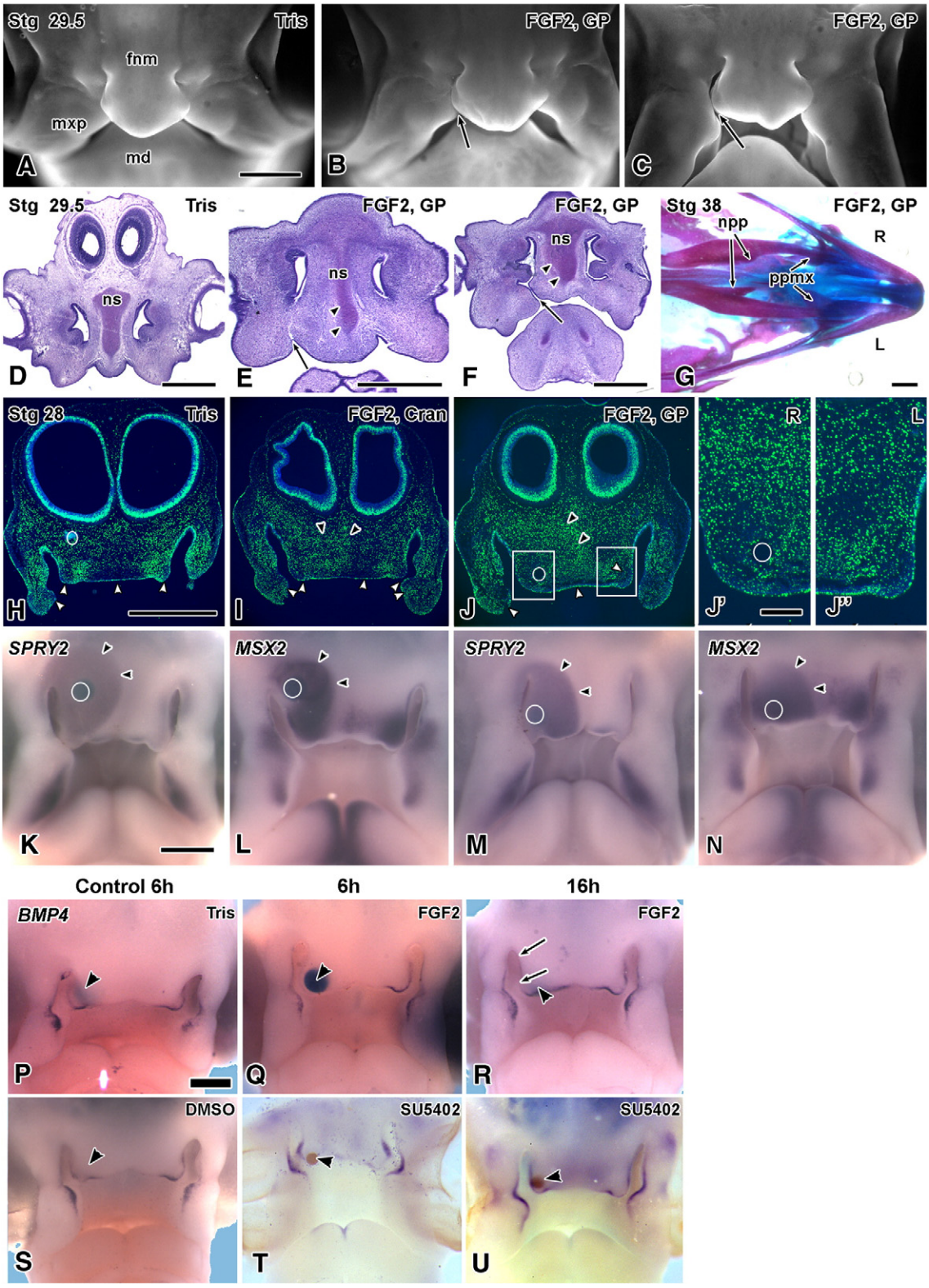
We looked at the effects on the skeleton to see whether the foil had interfered with frontonasal mass morphogenesis. Externally the foil implanted embryos did not have clefts in the side of the beak as with the SU5402 embryos, however internally, there was an obvious shortening of the maxillary process of the premaxillary bone on the treated side in all but one of the embryos (n=12/13, Figs. 6C–F). This is almost identical to the SU5402 phenotype and suggests that the foil is not interfering with fusion but nonetheless has similar effects on skeletogenesis to the SU5402 beads. The reasons for the skeletal phenotype are unclear but may relate to the increase in *SPRY2* expression, perhaps resulting in a dampening of FGF signaling. In summary, the foil barrier experiments increased *SPRY2* expression but we needed further evidence to say whether FGF signaling was decreased or increased. We therefore performed the complementary, gain-of-function experiment in which FGF2 beads were implanted and compared the phenotypes.

Increased FGF signaling does not induce precocious fusion

We predicted that a local increase in FGF signaling, especially in the cranial, FGF-dependent position would induce a burst of proliferation and perhaps lead to abnormal or early contact between the globular process and maxillary prominence or nasal cavity, such early contact could lead to precocious fusion of the lip. However, we found that embryos that were treated in the cranial region of the frontonasal mass developed normally (n=19/22) and there was no evidence of precocious fusion in serially sectioned embryos at any of the timepoints examined (12 h, n=4; 56 h, n=8, Fig. 7I and data not shown). In contrast, the caudally treated embryos had either partial (Figs. 7B, E, F) or complete clefts (Fig. 7C, n=3/11). The early morphology produced by caudal FGF2 was a more rounded globular processes (n=19/33; compare Figs. 7A to B, C) an effect not seen with Tris-soaked beads (n=17/18; Fig. 7A). In the material examined, we did not detect ectopic contact between the frontonasal mass and the nasal cavity, however it is possible that due to the complex nature of the nasal passages some small areas of contact with the maxillary prominences could have been present. The most obvious difference in the FGF2 treated tissue was that the nasal cartilage was often displaced to the left (n=9/11, Figs. 7E, F) possibly due to asymmetric

proliferation in the mesenchyme. There were no effects on epithelial thickness in either cranial or caudal FGF2 bead implants. To determine whether these early effects on early morphology had any consequences on skeletal development, we allowed a series of embryos to develop until the beak had fully formed. We found that the FGF2 soaked beads placed in the caudal bead position reduced the outgrowth of the upper beak, making it slightly shorter than the lower beak (n=7/13), and in a few cases resulting in minor clefts between the premaxilla and palatine process of the maxillary bone (affecting the roof of the mouth and not the side of the beak) however these were much less severe than those reported for SU5402 (n=7/13, Fig. 7G). Thus embryos had a different phenotype from either those produced by SU5402 or foil-barriers.

To determine whether the shape changes in the FGF2-treated embryos were due to an increase in proliferation, we looked at BrdU incorporation. There were many more cells labeled in the treated



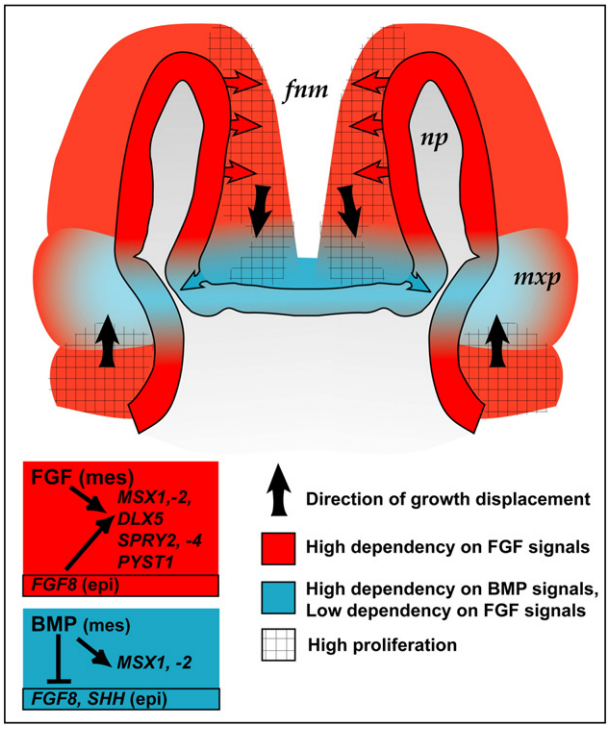


Fig. 8. Regions of FGF dependence and independence in the developing face. FGF signaling (red) from upper frontonasal mass (mesenchymal and epithelial) promotes proliferation and cell survival. Displacement of the globular process is caused by expansion and relatively higher proliferation of the cranial frontonasal mass mesenchyme (black arrows). FGF signaling is less important in the globular process and cranial maxillary prominence. In these two regions BMP in the mesenchyme positively regulates *MSX1* and *MSX2* expression and represses *FGF8* and *SHH* in the epithelium (Ashique et al., 2002a). Areas of high proliferation overlap all of the FGF-dependent regions and also a small area in the caudal edge of the frontonasal mass that is dependent on BMP signals.

side than the untreated side of the frontonasal mass in both cranial (Fig. 7I) and caudal bead positions (Figs. 7J–J) The expansion of mesenchyme at this early timepoint (12 h post bead implantation) would undoubtedly the cause of the deviated nasal cartilage seen at later timepoints (Figs. 7E, F) There was also a clear increase in the central frontonasal mass (Figs. 7I–J′), an area that we have previously defined as having relatively low proliferation in the normal embryo (Fig. 7H; MacDonald et al., 2004). Remarkably however, the beads did not increase proliferation within the globular process, even though the beads were placed in direct contact with this mesenchyme (Figs. 7J, J′, $n=5/5$). Moreover the FGF2 protein was able to stimulate proliferation in the cranial frontonasal mass ($n=5/5$; Figs. 7I–K) an area we had shown to be FGF-dependent. The responses to FGF2 are therefore similar to those obtained with SU5402 and further highlight the independence of the globular process from FGF signaling.

The previous experiments with SU5402 had shown *SPRY2* and *MSX2* to be targets of the FGF pathway. As expected, there was significant upregulation in embryos treated with cranial (Figs. 7L, M, $n=4/4$ for *MSX2* and 6/6 for *SPRY2*) or caudal beads (Figs. 7N,O $n=5/5$ for both genes) that extended many hundreds of microns away from the bead. The negative feedback loop where the agonist FGF induces its antagonist *SPRY2* is similar to that reported by others (Chambers and Mason, 2000). There was a striking correlation between the areas with increased proliferation and increased gene expression with the exception of the globular process. As described previously, proliferation in the globular process remained low even though FGF2 induced expression of *SPRY2* and *MSX2*. We therefore hypothesized that *BMP4*, which is expressed in the globular process and is required for proliferation in this region (Ashique et al., 2002a) was decreased in response to FGF2.

BMP4 expression is largely complementary to that of *FGF8*, a pattern that is repeated in the mandibular and maxillary prominences. As a consequence of conditional deletions of *Bmp4* there often is an expansion of *Fgf8* (Liu et al., 2005), therefore reciprocal regulation is thought to be taking place. The prediction is that *BMP4* should be repressed by FGF2 and increased by SU5402. Indeed *BMP4* expression was completely lost at 6 h after FGF2 beads were implanted ($n=4/4$, Fig. 7M). The downregulation was maintained in the nasal slit (arrowhead, $n=3/3$, Fig. 7N) but not the caudal edge epithelium 16 h after implantation. It is possible that the loss of *BMP4* helps to keep proliferation low in the globular process. We compared these FGF2 data to the effects of SU5402 and neither the caudal (Figs. 7P, Q; 6 h, $n=5/6$; 16 h, $n=6/6$) nor cranial SU5402 beads (data not shown; 6 h, $n=5/5$; 16 h, $n=5/5$) affected *BMP4* expression. Therefore there is a feedback loop such that FGF signaling is not required to maintain *BMP4* expression but instead FGFs may act to restrict *BMP4* to specific regions of facial ectoderm.

Discussion

Here we have shown novel roles for FGF signaling in the face including the regulation of shape and volume in the facial prominences. Furthermore, through our analysis of the response to the cranial and caudal bead positions we localized the areas of greatest FGF responsiveness to the cranial and lateral edge of the frontonasal mass, whereas the globular process and cranial maxillary prominences are not dependent on FGF signalling. Together these regions are coordinated so that the physical association of the facial prominences occurs at the correct time and place of development. The nasal slit epithelium, which expresses both *FGF8* and *FGF10,* is one important source of FGF signals.

Loss of FGFR signaling gives rise to the skeletal deletions

The SU5402 experiments led to a net reduction of endogenous FGF, even though there are decreased transcripts for intracellular inhibitors of the FGF signal (*SPRY2, 4*, *PYST1*). These data are further supported by the loss of Phospho-Erk staining and the decreased expression of 2

Fig. 7. Effects of exogenous FGF2 on morphogenesis, proliferation and gene expression. Embryos treated with either Tris buffer-soaked (A, D, H, L), FGF2-soaked (1 mg/ml; B, C, E–G, I–K,M, N) or SU5402-soaked beads (P, Q). (A, D) Normal morphology at 55 h showing narrow frontonasal mass. (B, C) An increase in size of the right frontonasal mass (arrow). The embryo in B is less affected than in C. The embryo in C has a cleft (arrow) and looks similar to a duck embryo of an equivalent stage (Wu et al., 2006). (D–F) Frontal sections of embryos 55 h post bead implantation, stained with Toluidine blue. (E, F) Two near adjacent sections from the embryo in panel B displaying a thinner fusion zone (arrow) and communication between the nasal and oral cavities (arrow). The prenasal cartilage is displaced towards left (arrowheads). (G) Palatal view of a caudally implanted FGF2 embryo at stage 37 showing reduction of the palatine process of the maxillary bone and nasal process of the palatine bone. (H–J) BrdU labeling 12 h after bead implantation, showing areas of low proliferation in the zone of fusion and along the caudal edge (white arrowheads) and high proliferation in the centre of the frontonasal mass (black arrowheads). Beads indicated with white circles. The globular process and caudal edge of the frontonasal mass of treated embryos have low proliferation similar to controls. Higher power views show no change in proliferation in the globular process epithelium compared to the untreated (left) side. (K–N) FGF2 beads activate target genes 16 h post bead implantation (arrowheads). (P–R) *BMP4* expression is eliminated at early timepoints and recovers along the caudal edge by 16 h (continued loss of expression in nasal slit is indicated by arrows). Note swelling of globular process in panels N. (S–U) SU5402 had no effect even though the bead was directly in contact with expressing epithelia. Scale bars = 1 mm for panels A–K, 200 μm for panels J′, J and 500 μm for panels K–U. Key: fnm, frontonasal mass; gp, globular process; md, mandibular prominence; mxp, maxillary prominence; npp, nasal process of palatine bone; ns, nasal septum; ppmx, palatine process of maxillary bone.

downtstream targets of FGF (*MSX1, MSX2*). Our skeletal results are consistent with a loss of FGF signaling and are similar to the results of gene targeting of *Fgf* ligands in mice. The conditional knockouts (Kawauchi et al., 2005; Macatee et al., 2003; Trumpp et al., 1999) and hypomorphic alleles of *Fgf8* (Abu-Issa et al., 2002; Frank et al., 2002) have reduced maxillary and premaxillary bones, coincident with the foreshortening of the upper face (Kawauchi et al., 2005; Macatee et al., 2003). Although no cleft lips were observed in the various FGF knockout mice, the losses of distal facial bones are similar to the loss of bones in cranially treated SU5402 treated embryos.

What is the interpretation of the foil barrier data then, in which *SPRY2* expression is increased and yet a phenotype almost identical to SU5402 is produced? There are two possibilities to consider. One is that the increased *SPRY2* lead to a decrease in the level of FGF signaling. Although the foil has allowed the FGF protein to build up in the lateral edge of the frontonasal mass, the concomitant upregulation of *SPRY2* leads to a reduction in FGF signalling. Further evidence in support of this idea is that the foil experiment gives different results to those obtained with FGF2 bead implants.

Another possibility is that the tissues medial to the foil are prevented from receiving an FGF signal and it is these regions that give rise to the lateral process of the premaxilla. There are no precise maps of the frontonasal mass in which specific skeletal elements are mapped onto a stage 24–28 frontonasal mass, however there are data showing that the lateral edge and globular process of the frontonasal mass contribute to the sides of the upper beak (Hu et al., 2003). The centre of the beak is derived from cells in the midline and at the caudal edge of the frontonasal mass. Based our knowledge of the upper beak skeleton in relation to the overlying soft tissues, we can deduce that the centre of the beak is derived from cells in the midline and at the caudal edge of the frontonasal mass whereas the maxillary process of premaxilla originates in the lateral edge of the frontonasal mass. We correlate the region in which *SPRY2* was upregulated in the foil implants with the mesenchyme that gives rise to the maxillary and palatine process of the premaxilla. Dil labeling of the frontonasal mass showed that cells labeled in the area closest to the foil position mainly expand vertically and not as much laterally (McGonnell et al., 1998). Therefore we should not have interfered with the normal cellular movements at that stage. Work from others has shown that, retroviral over-expression of *SPRY2* in the face lead to a cleft beak with major loss of skeletal elements (Goodnough et al., 2007). Thus we conclude that *SPRY2* is one gene expressed in the right place and right time to affect skeletogenesis in foil implanted embryos but we cannot rule out that additional genes mediate this process.

In summary, the foil barriers can phenocopy some of the effects of cranial SU5402 beads and it is primarily on this basis, that we conclude foils are disrupting FGF signaling, possibly via upregulation of the antagonist, *SPRY2*.

Hierarchy of signaling in facial prominences shows BMPs to be acting upstream of FGFs

The cranial and lateral edges of the frontonasal mass, caudal maxillary prominence have high *SPRY* expression and are flanked by zones that are largely controlled by BMP signals. BMP-dependence has been demonstrated in both mouse and chicken embryos. For example, conditional deletion of *Bmp4* in the mandibular epithelium specifically disturbed distal development (Liu et al., 2005), the area spared in the *Fgf8* conditional knockouts (Trumpp et al., 1999). Furthermore, our previous studies on chicken showed that endogenous BMPs are required for development of the globular process and anterior maxillary prominence (Ashique et al., 2002a). The primary roles of endogenous Bmps in the globular process are to 1) regulate proliferation and 2) to regulate thickness of the epithelia which must be thinned and removed for a mesenchymal connection to form (Ashique et al., 2002a). This latter conclusion is based on the thickening of epithelium and upregulation of *FGF8* following treatment of the globular process with Noggin. In contrast, in the present study, FGFs do not appear to be involved in regulating epithelial survival since exogenous FGFs do not result in detectable thickening of the epithelium in tissue sections.

Previous data with Noggin beads (Ashique et al., 2002a) has shown that BMPs are upstream of *Fgf* genes and that normally the role of BMPs is to repress *Fgf8* expression in the globular process. In the mouse model, conditional deletion of *Bmp4* in the mandibular epithelium expands *Fgf8* expression towards the midline, an area that normally expresses *Bmp4* (Liu et al., 2005). Therefore not only are BMPs upstream of FGFs, the chicken and mouse data agree that the role of BMPs is to repress the expression domain of FGF8. We extend these results here and show that blocking FGF signalling had no effect on *BMP4* expression, confirming in a complementary experiment that FGFs are not upstream of BMPs. Once again, these results on chicken are similar to those obtained in the *Fgf8* conditional knockout mice, where there is no effect on *Bmp4* expression in the mandibular prominence (Trumpp et al., 1999).

It is also interesting that the same transcription factors lie downstream of BMP and FGF signaling, *MSX1*, *MSX2*. We have shown that *MSX2* transcripts in the globular process are dependent on BMP signaling (Ashique et al., 2002a) and FGF signaling. However during normal morphogenesis FGFs are not active in the globular process and BMPs take over the role of maintaining *MSX2* expression. *MSX2* is associated with cleft lip in humans (van den Boogaard et al., 2000) and now we have shown that disruptions in either the FGF or BMP signaling pathways can affect MSX2 and give rise to a cleft. These data demonstrate that there is crosstalk between FGF and BMPs and their downstream effector molecules in the globular process.

The chicken embryo data provide experimental validation and a mechanism for how FGF signaling is used during human lip fusion

The data presented here showed that mainly it is decreased FGFR signaling in the lateral edge of the frontonasal mass that induced a cleft lip. Those clefts induced by FGF2 are minor and for the most part embryos recover and develop normally from this insult. In the case where there is too little FGF signaling, contact between adjacent prominences is not made at the critical time and this is the principal reason for the failure of fusion. Such studies are relevant to human clefting because promoting better growth in the early phases of facial development would facilitate fusion. Further explorations into the precise timing, size and shape analysis of the facial prominences during upper lip fusion (Diewert and Wang, 1992; Diewert et al., 1993; Wang et al., 1995) will provide valuable insights into spectrum of severity within human orofacial clefting.

The association between FGF signaling and orofacial clefting in humans has recently been solidified due to identification of loss-of-function mutations in FGFR1 in Kallmann syndrome, which has cleft lip as part of the phenotype (Dode et al., 2007; Dode et al., 2003) and the finding of missense mutations in various FGF pathway genes in populations with isolated clefts (Riley et al., 2007a). Kallmann syndrome is particularly interesting to us because patients present with anosmia as well as cleft lip. Our data from extirpation experiments and foil barriers showing that nasal slit epithelium provides at least some of the FGF present in the frontonasal mass provides a mechanism for how nose and facial defects could arise together. It is very intriguing that the Twirler mouse also has a combination of cleft lip and olfactory nerve deficits (Gong, 2001; Gong et al., 2000). The gene mutated in Twirler mice has not been identified but genes in the FGF pathway that occur in the affected locus should be examined carefully.

The FGF pathway genes with variant alleles in populations with isolated CL/P (cleft lip with or without cleft palate) include *FGF8, FGFR1, FGFR2*, and *FGFR3* (Riley et al., 2007a). In addition, genome-

wide screen identified the locus 8p11 as being a hot spot for genes that are linked to clefting. FGFR1 is contained within this region (Riley et al., 2007b). Moreover, several of the genes found to be regulated by FGFs in our study such as *MSX1*, *MSX2* and *SPRY2* have also been linked to non-syndromic human CL/P (Jezewski et al., 2003; Suzuki et al., 2004; Vieira et al., 2005). Our work provides the first experimental evidence that loss of FGF signaling from the cranial region of the frontonasal mass disturbs outgrowth. Our data from FGF2 bead implants, also suggest that gain-of-function mutations in FGF signaling could lead to craniofacial abnormalities.

Growth factor signalling and morphogenesis of species-specific form

The morphogenesis of species specific form is dependent on coordination of growth factor signalling in different regions of the face as proposed by Chuong and colleagues (Wu et al., 2006). In their model the chicken beak has higher proliferation in the corners and this is followed by convergence of the two lateral domains into one area of high proliferation in the centre of the beak (Wu et al., 2006, 2004). At an equivalent morphological stage, these authors found that the broad-billed duck does not lose the higher proliferation at the corners, thereby it has a wider frontonasal mass. It is interesting that we have converted the morphology of the chicken frontonasal mass to be more duck-like with FGF2 beads placed in the globular process and this morphology change is due to increased proliferation. Since the effects from protein-soaked beads are unilateral and transitory, increases in beak width were not seen. Nonetheless, we agree with the model proposed by Wu and colleagues that higher lateral proliferation leads to a broader morphology (Wu et al., 2006).

FGF-dependent regions are the main growth centres of the face

The placement of SU5402 beads showed a high requirement for FGF signalling in the lateral frontonasal mass corresponding to areas of high BrdU labeling but not medially where low BrdU labeling is found. These lateral regions are also very responsive to exogenous FGFs. We propose that most of the growth in the frontonasal mass occurs in the lateral edges of the frontonasal mass under the influence of the FGF-rich nasal slit (Fig. 8). This results in caudal or vertical displacement of the globular process and edge of the frontonasal mass.

We have uncovered two things in this study: the signaling molecules that contribute, and a biomechanical explanation for the clefting phenotype. The cleft embryos in this study had a reduced volume within the cranial frontonasal mass, which prevented the vertical displacement of the globular process towards the anterior maxillary prominence.

The displaced regions are mainly dependent on other growth factors and less so on FGFs. For example, near the edge of the frontonasal mass there is high expression of *BMP7* and *BMP4* (Ashique et al., 2002a,b). Furthermore, when BMP signaling is blocked with Noggin protein there is a reduction in the already low rate of proliferation and clefts are induced (Ashique et al., 2002a). Thus our model of facial morphogenesis is comprised of FGF signalling mainly occurring in the lateral and cranial edges of the frontonasal mass, whereas the centre and globular processes are BMP independent (Fig. 8).

We proposed a modification of the beak morphogenesis model to say that FGF is also a major signal controlling midfacial growth. We have also refined the model to locate these different FGF and BMP-dependent domains in relation to areas of high and low proliferation (Fig. 8). Such experimentation using the highly varied bird beak and other animal models with divergent facial morphologies will reveal more about the universality of these mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.027.

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